Semicontinuous nitrogen limitation as convenient operation strategy to maximize fatty acid production in Neochloris oleoabundans

UNIVERSITÀ DEGLI STUDI DI TORINO

Semicontinuous Nitrogen limitation as convenient operation strategy to maximize fatty acid production in *Neochloris oleoabundans*

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Abstract

Neochloris oleoabundans is a green microalga known for the ability to increase its fatty acid (FA) content when grown under nitrogen (N) depletion. Accumulation of FAs and their composition were compared by cultivating *N. oleoabundans* in N limitation and N depletion conditions. We adopted an innovative approach that consists in semicontinuous cultivation mode as a strategy to optimize lipid accumulation. To identify the optimal conditions for the production of different FAs, a temporal profile of how different conditions may influence the microalgae culture in terms of biomass, FA concentration and composition was evaluated. N limited culture attained higher values of algal productivity and total FA concentration (0.42 mg L^{-1} d^{-1} and 91.2 mg L^{-1}) than N deplete culture (0.15 mg L^{-1} d^{-1} and 53.2 mg L^{-1}). Considering lipid accumulation, concentration and percentage of triacylglycerols (TAG) increased in all culture methods, with respect to the N replete control. In particular, N limitation led to a significant increase of polyunsaturated TAG (PUFA), while N depletion led to the highest level of monounsaturated TAG. These results suggest that *N. oleoabundans* biomass and FAs were strongly affected by the N supply and that the quality of the TAG fraction could be modulated in accordance to the utilization purpose in a wide range of applications. Semicontinuous culture resulted as a promising operational strategy for FAs production. More specifically, semicontinuous cultivation coupled to N limitation was found to be a convenient method to increment PUFA fraction and therefore to yield a high value product.

Key-words: biomass, fatty acids, triacylglycerols, lipids, microalgal cultivation, algal productivity

1. Introduction

The use of microalgae has several advantages when compared to other available feedstock (e.g., soybean, rapeseed, sunflowers and palm oil) [1]. Microalgae have higher growth rates and areal productivities of biomass and lipids than conventional crops, which eventually results in a lower demand of land area. Furthermore, since microalgae can be cultivated in non-arable land, they do not compete with agriculture. Microalgae cultivation is not seasonally limited and allows daily harvests [2]. Moreover, other compounds can be extracted from microalgae residual biomass such as polyunsatured fatty acids (PUFAs), sugars, pigments and antioxidants, that can be used in many commercial applications such as cosmetics, pharmaceutical and nutraceutical industries [3].
Recent studies highlighted that oleaginous algal strains cultivated under nitrogen (N) deficiency increase their lipid content without a substantial decrease in biomass productivity [4,5] . Most studies report data in terms of maximum lipid content, without a correlation with the biomass productivity and without considering that the maximum lipid content is often attained when the biomass productivity is at a minimum. Instead, a key-parameter to evaluate lipid production should be lipid productivity [6], meant as the product of biomass productivity and the lipid content. In addition, lipid composition is an important aspect to be evaluated, because different fatty acids (FAs) affect the quality of the final product. Depending on the use of the oil (energy production, food or feed), the required characteristics may vary. For biodiesel production, a high presence of monounsaturated FAs (MUFA as oleic and palmitoleic acids), a reduced presence of PUFA, and a controlled saturated FA content are recommended [7,8]. On the other hand, PUFA, especially Ω-3 PUFA, have been shown to be effective in preventing or treating several diseases [9,10,11,12]; therefore, this is the most interesting FA fraction for human as well as for animal nutrition [13,14].
Different cultivation conditions may affect the lipid content and the FA composition; under N deficiency, the protein content and the chlorophyll level decrease, while carbohydrate and lipid content increase [15,16]. A higher lipid content is mainly due to an accumulation of triacylglycerols (TAG), which are the preferred substrate for biodiesel production. In green algae, the variation in the FA composition usually results in increased oleic acid contents, with a consequent decrease in the average degree of polyunsaturation [17,5]. The cultivation of microalgae under N stress is carried out either by N depletion or N limitation. Under N depletion, microalgae grow in a medium lacking a N source, while under N limitation there is a constant but insufficient supply of N. Using N depletion to increase the lipid content in algae, especially TAG fraction, is a well- known process [17,2,18,19,4,5]; whereas N limitation is less studied or it is meant as progressive depletion [4,20,21]. *Neochloris oleoabundans* is a microalga also known for its potential in biodiesel production [17, 22,23,18,24]. This species when grown in batch N depletion may reach a total lipid content up to 40% [22,18], and has been tested in a wide range of cultural conditions [25,26].
The aim of the present study is to investigate the effect on biomass growth, lipid production and FA composition of *N. oleoabundans* under N depletion and N limitation. While other studies have investigated the effect of the culture conditions on the final lipid content, lipid and biomass concentration and the temporal trend of these parameters were seldom reported. Here, a temporal profile of how different conditions (sufficient/limited/deplete) may influence the microalgal culture in term of biomass, lipid concentration and FA composition is reported, with the aim to define the optimal conditions for the production of different lipids.

2. Materials and methods

2.1 Pre-cultivation conditions

Neochloris oleoabundans UTEX 1185 was obtained from the University of Texas Culture Collection of Algae (UTEX). *N. oleoabundans* was inoculated in 250 ml Erlenmeyer flasks

containing 100 ml of liquid medium. Flasks were maintained in sterile condition in a CO_2 incubator (Sanyo CO_2 Incubator Mco-19Aic) flushed with air/CO_2 (97/3, v/v) to support growth and maintain pH within a desired range. In the incubator, the temperature was 25 ± 2°C and a continuous artificial illumination of 200 µmol m^{-2} s^{-1} was provided by daylight LEDs. To allow mixing of the culture an orbital shaker with 150 rpm rotation speed was used.
The culture medium was a modified BG11 medium (MBG11), which was composed of (in mg L^{-1}): 1500 $NaNO_3$, 20 Na_2CO_3, 140 K_2HPO_4, 135 $MgSO_4 \cdot 7H_2O$, 47 $CaCl_2 \cdot 2H_2O$, 6 citric acid, 25 Fe (III) NH_4 citrate, 1 Na_2EDTA, 2.86 H_3BO_3, 1.81 $MnCl_2 \cdot 4H_2O$, 0.39 $Na_2MoO_4 \cdot 2H_2O$, 0.222 $ZnSO_4 \cdot 7H_2O$, 0.079 $CuSO_4 \cdot 5H_2O$, and 0.049 $CoCl_2 \cdot 6H_2O$. The medium pH was then adjusted with 1 M HCl to pH 7.0 and autoclaved.

2.2 Test of different culture conditions (N replete/limited/deplete)

Tests at different N concentrations were performed in semicontinuous modes.
Experiments were carried out in 800 ml glass bubble tubes containing 400 ml culture. The daily dilution rate was 50%, replaced with an equal volume of MBG11 medium containing different N concentrations, depending on treatments. MBG11 medium was used in N replete (R). In N limitation (L), the N concentration was 10% with respect to R (24.75 mg L^{-1}); while in N depletion (D) the MBG11 was used with an initial N concentration of 24.75 mg L^{-1} and was no longer added for the rest of the experiment.
A continuous flow of air:CO_2 (97:3 v/v) was provided in order to control pH, ensure CO_2 sufficiency and mix the culture; moreover, the temperature was maintained at 28 ± 2°C with an average continuous light intensity of 300 µmol m^{-2} s^{-1}. The pH was measured every day and the bubbling of CO_2 was regulated (range between 3 and 3.5%) in order to maintain a pH in the 6.5-7.5 range. Inocula for all the experiments were obtained by centrifugation (2000 rpm for 30 min) of biomass grown in flasks maintained in the incubator and subsequently re-suspended in a medium necessary for the experiment. For all tests, the initial biomass concentration was 0.7 g L^{-1} (dry weight). The test cultivation time was 7 days. All experiments were repeated in triplicate.

2.3 Culture analysis

Samples for dry weight (dw) calculation were taken daily in triplicate and a gravimetric determination was performed according to [27]. Samples for lipid extraction were taken from the cultures at day 0, 4 and 7, lyophilized and stored at -20°C.

2.4 Lipid extraction and transesterification

Lyophilized algae were extracted with a Soxhlet apparatus by using $CHCl_3$-hexane (2:1 v/v). The extract was evaporated under N_2 and weighted. The extract was resuspended in hexane and then separated according to [28], by TLC in two runs: (i) hexane-diethyl ether-acetic acid, 70:30:1; (ii) hexane-diethyl ether, 95:5. After spraying with 2'-7'-dichlorofluorescein, TLC plates were observed under UV (385 nm) and spots corresponding to standards of free fatty acids (FFA; heptadecanoic acid), monoacylglycerols (MAG; monopalmitin), diacylglycerols (DAG; dilinolein) and triacylglycerols (TAG; trilinolein) were scraped off and extracted with $CHCI_3$. Fatty acid methyl esters (FAME) were obtained, after addition of an aliquot of the internal standard heptadecanoate, by treatment with MeOH-BF_3 according to the method described by [29].

2.5 Gas Chromatography and mass spectrometry

The quantitative determination of FAME from FFA, MAG, DAG and TAG was obtained by gas chromatography by using a flame ionization detector (FID-GC 6890N, Agilent Technologies). A ZB5-MS 30-m column (Zebron 7HG-G010-11, Phenomenex) was used with the following temperature program: 60 °C for 1 min then an increasing rate of 10 °C min^{-1} up to 180 °C, a second increase of 1 °C min^{-1} up to 230 °C, then 15 °C min^{-1} to reach 290 °C. The injector temperature was 280 °C and the detector 280 °C; the carrier gas was He with a flow rate of 1 ml min^{-1}; splitless injection mode. Based on internal standard area, FAME from FFA, MAG, DAG and TAG were quantitatively estimated on an algae dry weight basis.

Compounds were identified by both retention times, comparison of pure standards and gas chromatography coupled to mass spectrometry (GC-MS, GC 6890N, MS 5973N, Agilent Technologies). Carrier gas was He with a constant flow of 1 mL min^{-1}, transfer line temperature to MS Detector was 280°C, ionization energy 70 eV, and full scan range 50–500 *m/z*.

The following abbreviations were used for the identified FA: C16:0= palmitic acid; C16:1= palmitoleic acid (cis-9-hexadecenoic acid); C16:2= palmitolenic acid (all-cis-9,12-hexadecadienoic acid); C16:3= all-cis-7,10,13-hexadecatrienoic acid; C18:0= stearic acid; C18:1=oleic acid (cis-9-octadecenoic acid); C18:2= linoleic acid (all-cis-9,12-octadecadienoic acid); C18:3= linolenic acid (all-cis-9,12,15-octadecatrienoic acid).

2.6 Calculations

Biomass productivity (mg L^{-1} d^{-1}) was calculated as the change in concentration (mg L^{-1}) between two consecutive sampling times; specific growth rate (μ) was calculated as the change in biomass concentration (expressed as natural logarithm) as a function of time. The doubling time (T_d) was calculated by dividing ln2 by the growth rate. Total fatty acids concentration (mg L^{-1}) was calculated as the product of biomass concentration (g L^{-1}) and total fatty acids content (TFA) (mg g^{-1} of biomass).

2.7 Statistical analyses

All data are the mean of at least three replicates. ANOVA and Tukey-Kramer's HSD test ($p<0.05$) were used to determine significant differences among different cultivation conditions on data concerning biomass, productivity and FA composition using the SYSTAT 10 software.

3. Results and discussion

3.1 Biomass concentration

Biomass concentrations of microalgae growth in N replete/limited/deplete conditions are shown in Figure 1.

Insert Fig. 1 here

As expected, the highest biomass concentration was achieved in N replete condition. After the first 3 days of adaptation phase, in N replete and limited cultures the daily productivity compensates the daily harvesting, thus keeping constant the algal concentration at the sampling time. This condition was maintained throughout the experiment by a constant N supply (Fig. 1a). The deplete culture

never reached this equilibrium due to a progressive N depletion that prevented a constant increase of biomass concentration (Fig. 1b). Indeed, in the last days of depletion the dilution rate was higher than the specific growth rate, thus the system wash out gradually occurred. At the onset, the biomass concentration of deplete and limited cultures was similar. However, with increasing time, the absence of an external N source slowed the algal growth in deplete culture, and after the 2nd day the biomass rapidly decreased. These results showed that culture conditions significantly ($p<0.05$) affected algae growth. Maximum, minimum and mean daily biomass productivities, growth rate and doubling time calculated for the duration of the experiments are reported in Table 1. Standard deviations are reported for all parameters except for the doubling time, which derives from the growth rate.

Insert Table 1 here

In replete conditions, the values obtained are comparable with those found in recent studies [30,26,31]. As observed with biomass values, maximum, minimum and mean productivities and growth rates increased along with N supply (Table 1). The only exception was the maximum biomass productivity, that was higher in N depletion than in N limitation, but this value was obtained at the onset, when the depletion was not effective yet. Biomass productivities were significantly different among the three cultivation conditions ($p<0.05$). The differences among them were even more evident in terms of cumulate biomass (g). The latter was calculated by adding the biomass harvested daily (Fig. 1b). Values reported were calculated by considering 1 L of initial volume.
The biomass productivity value obtained under N limited conditions was higher than that obtained by [32], who reported a biomass productivity of nearly 0.3 g $L^{-1}d^{-1}$. Most literature data refer to batch conditions. For example, Gouveia and Oliveira [2] found an average biomass productivity of 0.09 g L^{-1} d^{-1} testing *N. oleoabundans* in polyethylene bags under replete conditions using a light intensity of 150 μmol m^{-2} s^{-1}. Breuer et al. [5] cultivated *N. oleoabundans* in N replete and deplete conditions when the culture was maintained in flask in batch mode; they found a much higher biomass productivity compared to [2] and a low difference between the replete and the deplete conditions (0.45 g L^{-1} d^{-1} and 0.43 g L^{-1} d^{-1} respectively).

3.2 Total Fatty Acids production

Deplete cultures achieved the highest TFA contents (13.0 and 47.1% of biomass after 4 and 7 days respectively, Table 2). For the same strain, in [26] it is reported a TFA content of 18.1-18.5% in N depletion at pH 8.2 with a significant increase (29.2%) at pH 10. In enriched natural seawater, Popovich et al. [30] found a lipid content of 26.7% after six days of N depletion. For *Nannochloropsis,* values ranging from 14.7 up to 32.5% in a similar temporal N deprivation were obtained in [4]. In our study, limitation showed a considerable increase of TFA content during time (1.6, 5.1 and 11.1% at day 0, 4 and 7 respectively, Table 2). When only TFA production is considered, depletion could appear as the preferred method to produce TFA, but this outcome is reversed when biomass and total productivity data are considered. Until the 4th day, the reduction of biomass production in deplete culture did not affect TFA concentration, but at the 7th day the influence of the biomass increased, causing an increase in TFA concentration (91.2 mg L^{-1}) in limited culture (Fig.2), which was nearly 8 times higher than the initial concentration. Also Santos et al. [33] found the highest TFA content (42.4%) under the highest stress level (pH 10, N depletion and high irradiance), but the highest TFA productivity (176 mg $L^{-1}d^{-1}$) at a lower stress level (pH 8, N depletion and high irradiance). Our results showed that limitation optimizes biomass and TFA production. Therefore, semicontinuous culture process positively affected lipid accumulation, in accordance with [32], in which *Chlorella* sp. was cultivated under batch urea progressive depletion and semicontinuous urea limitation. They found a higher lipid production using semicontinuous

mode rather than using batch or fed-batch modes. The semicontinuous mode achieved the highest lipid productivity when compared to previous studies that were mostly focused on batch cultivation with N depletion. Under those conditions, Takagi et al. [34] demonstrated that limitation achieved a higher lipid and TG content compared to progressive depletion in *Nannochloris* sp. A further increase in TFA production can be attained optimizing other parameters (nutrient concentrations, dilution rates, irradiance) as performed in [35] using the marine *Nannochloropsis gaditana*.

Insert Fig. 2 here

3.3 Fatty Acids composition

3.3.1 Triacylglycerols

In accordance with previous works [23,30], the general FA composition in the TAG fractions of *N. oleoabundans* had a higher content of oleic acid (C18:1) followed by palmitic (C16:0) and stearic (18:0) acids (Table 3); however, some differences among culture methods could be observed.
In nitrogen limitation conditions the saturated fraction of triglycerides decreased significantly in favor to MUFA and, in particular, PUFA (1.6 and 1.5 times at 4 and 7 days, respectively, Table 3). This trend was not observed in nitrogen replete. In nitrogen depletion conditions, saturated and polyunsaturated fatty acids slightly but significantly decreased in favor to the MUFA fraction. Regarding triglycerides quantity, the highest value was reached after 7 days of nitrogen depletion (344.39 mg g^{-1}). After 7 days of nitrogen limitation, significantly similar values were obtained with respect to the 4^{th} day of depletion (85.01 and 88.24 mg g^{-1} respectively).

3.3.2 Free fatty acids

After 7 days of cultivation, with respect to the nitrogen replete, limited and deplete cultures showed a significant decrease of PUFA and MUFA in favor to saturated fatty acids. As for TAG, after 7 days the highest FFA content was also observed under nitrogen depletion (57.28 mg g^{-1}, Table 4).

3.3.3 Monoacylglycerols and Diacylglycerols

The percentage of the MAG fraction did not increase with respect to the controls during the cultivation period (Table 4). At the 4^{th} day in both treatments, DAG fraction percentage increased (2.7 and 2.1 times for limitation and depletion respectively); however, at the 7^{th} day values decreased as reported in Table 2. In both N-limited and deplete cultures, a decrease of the DAG fraction precursors (FFA and MAG) was observed at 4 days of treatment. After 7 days, DAG percentage decreased in favor of TAG accumulation.
Considering the percentage of TAG with respect to the sum of the other fractions (DAG, MAG and FFA) (Table 2), the overall ratio increased in all treatments with respect to the N replete control. In particular, the highest percentage values were observed under nitrogen limitation after 7 days (76.4%) with respect to nitrogen depletion (73.2%). These values are comparable to the 78.2% of TAGs found in [30] by cultivating *N. oleoabundans* in batch mode under nitrogen depletion (78%).
A different FA accumulation (higher under N depletion) might suggest a relation between lipid accumulation and the different cell culture density, as reported in [18]. Moreover, in [17] an increasing saturation level of FAs with increasing N limitation was reported. This was also observed in FFA fractions from saturated FA (32.4 - 38.5 %) in N replete cultures, to higher values observed in N limitation and N depletion (54.9 - 58.1 % and 54.5 – 71.5 %, respectively). An opposite trend was observed in the TAG fraction, where the FA saturation level decreases in favor of PUFA increase under N limitation. On the other hand, under N depletion an increment of monounsaturated FA was observed, as found in [30] in batch cultivation under N stress.
In general, the trends obtained with a semicontinuous cultivation technique and different N concentrations indicate that FA saturation can be influenced by N nutrition, affecting the total FA content.

Insert Table 2 here
Insert Table 3 here
Insert Table 4 here

4. Conclusions

This study shows that biomass and TFA are strongly affected by the cultivation method. TFA concentration was positively affected by N limitation, which maximized biomass production and TFA accumulation. Semicontinuous is confirmed as an effective cultivation method as it allows a greater availability of light per cell when compared to batch mode [18,19]. In semicontinuous culture, the biomass concentration can be controlled by varying the dilution rate and it can attain a constant biomass concentration for extended time.

With respect to N depletion cultures, N limitation and N replete under batch cultivation imply self shading limitation due to higher biomass concentration, whereas semicontinuous culture allows a greater light availability especially in N limited cultures. This can affect the biomass productivity as well as doubling time and other parameters.

Our methodological approach allowed us to highlight quantitative differences under different nutrition conditions; this can be a valuable information for the commercial use of microalgae for FA production. By differentially using these three cultivation methods, the quality of the TAG as well as the other fractions can be modulated depending on the utilization purpose.

Further optimization of semicontinuous cultivation, concerning for instance irradiance, dilution rates and culture media, may be a goal for future investigations.

5. Acknowledgements

This work was partly supported by Finpiemonte, project AlgaeNRG and by two grants from the Doctoral School of Sciences and Innovative Technologies, University of Turin. The authors are grateful to Rocco Mussat Sartor, Elena Ghiglione, Angelica Lucrezia Gibiino and Ambra Giuganino for their help in lipid extraction.

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Table 1

Biomass productivity (P), specific growth rate (μ) and doubling time (T_d) in N replete, limited and deplete cultures.

		N-Replete	N-Limited	N-Deplete
Biomass productivity (P)(g L^{-1} d^{-1})	Max	0.81 ± 0.06	0.56 ± 0.06	0.66 ± 0.07
	Min	0.59 ± 0.05	0.26 ± 0.06	-0.01 ± 0.01
	Mean	0.69 ± 0.08	0.42 ± 0.19	0.15 ± 0.02
Specific Growth rate (μ)		0.69 ± 0.05	0.58 ± 0.12	0.26 ±0.02
Doubling Time (T_d)		1	1.19	2.65

Table 2

Summary of fraction percentages and total fatty acids content (percentage on biomass dry weight). In the same row, different letters indicate significant ($p<0.05$) differences, while identical letters indicate grouping of values with no significant ($p>0.05$) differences.

		N Replete		**N Limited**		**N Deplete**	
	Time 0	**Time 4**	**Time 7**	**Time 4**	**Time 7**	**Time 4**	**Time 7**
TAG	68.9 ± 0.4 [a]	45.3 ± 0.2 [b]	22.6 ± 0.1 [c]	62.4 ± 0.3 [d]	76.4 ± 0.5 [e]	68.0 ± 4.0 [a]	73.2 ± 1.0 [a,e]
Free FA	17.6 ± 0.1 [a]	40.8 ± 0.3 [b]	43.7 ± 0.2 [c]	8.7 ± 0.1 [d]	6.3 ± 0.1 [e]	11.7 ± 0.1 [f]	12.2 ± 0.1 [g]
MAG	7.6 ± 1.1 [a]	6.1 ± 0.6 [a,c]	18.3 ± 1.7 [b]	7.2 ± 0.6 [a]	4.4 ± 0.4 [c,d]	3.2 ± 0.1 [d]	4.7 ± 0.6 [c,d]
DAG	6.0 ± 0.3 [a]	7.9 ± 1.1 [a]	15.4 ± 2.0 [b]	21.7 ± 2.2 [c]	12.9 ± 1.6 [b,d]	17.1 ± 1.5 [b]	9.9 ± 1.3 [a,d]
TFA	1.62 ± 0.03 [a]	2.40 ± 0.06 [b]	1.38 ± 0.06 [c]	5.08 ± 0.16 [d]	11.12 ± 0.27 [e]	12.98 ± 0.73 [f]	47.05 ± 1.36 [g]

Table 3

Triglycerides composition (values are expressed as µg or mg per grams of biomass dry weight). In the same row, different letters indicate significant ($p<0.05$) differences, while identical letters indicate grouping of values with no significant ($p>0.05$) differences.

		N Replete		N Limited		N Deplete	
FA (µg g^{-1})	**Time 0**	**Time 4**	**Time 7**	**Time 4**	**Time 7**	**Time 4**	**Time 7**
C16:0	3046.01 ± 21.71 [a]	2973.63 ± 8.84 [a]	844.41 ± 16.8 [b]	6281.79 ± 71.32 [b]	13497.47 ± 99.87 [b]	18590.2 ± 786.5 [b]	75722.99 ± 830.98 [b]
C16:1	388.66 ± 3.51 [a]	1015.56 ± 8.84 [b]	180.84 ± 2.58 [c]	302.57 ± 2.78 [b]	837.61 ± 3.63 [d]	762.9 ± 33.16 [d]	4127.34 ± 79.71 [e]
C16:2	7.93 ± 0.09 [a]	23.18 ± 0.15 [a]	4.25 ± 0.12 [a]	272.78 ± 1.97 [b]	862.62 ± 10.01 [c]	994.51 ± 107.39 [d]	2210.03 ± 39.5 [e]
C16:3	85.21 ± 0.72 [a]	97.16 ± 2.06 [a]	41.11 ± 0.4 [a]	297.74 ± 2.38 [b]	789 ± 15.68 [c]	493.68 ± 92.52 [d]	981.38 ± 17.9 [e]
C18:0	325.85 ± 2.1 [a]	692.96 ± 5.28 [a]	229.77 ± 11.52 [a]	1477.24 ± 12.57 [b]	3745.78 ± 26.71 [c]	3696.42 ± 187.48 [c]	39971.65 ± 492.77 [d]
C18:1	5359.46 ± 36.37 [a]	4427.61 ± 33.73 [a]	1335.6 ± 13.19 [a]	15408.82 ± 97.34 [b]	45332.1 ± 302.58 [c]	48488.76 ± 2935.41 [c]	180841.6 ± 3116.27 [d]
C18:2	1476.36 ± 8.02 [a]	1219.94 ± 12.26 [a]	370.72 ± 8.64 [a]	7276.59 ± 86.33 [b]	19035 ± 96.08 [c]	13978.53 ± 1200.19 [c]	31332.03 ± 242.65 [c]
C18:3	504.46 ± 2.26 [a]	424.83 ± 13.64 [a]	114.19 ± 1.39 [b]	396.95 ± 6.81 [a]	912.43 ± 6.61 [c]	1234.83 ± 169.48 [d]	9207.5 ± 68.46 [e]
Total (mg g^{-1})	11.19 ± 0.06 [a]	10.87 ± 0.05 [a]	3.12 ± 0.02 [b]	31.71 ± 0.14 [c]	85.01 ± 0.54 [d]	88.24 ± 5.17 [d]	344.39 ± 4.81 [e]
Saturation degree (percent values)							
Saturated FA	34.3 ± 0.2 [a]	33.7 ± 0.1 [b]	34.4 ± 0.1 [a]	24.5 ± 0.2 [c]	20.3 ± 0.1 [d]	25.3 ± 0.4 [e]	33.6 ± 0.1 [b]
Monounsaturated FA	46.8 ± 0.2 [a]	50.1 ± 0.2 [b]	48.6 ± 0.3 [c]	49.5 ± 0.1 [d]	54.3 ± 0.1 [e]	55.8 ± 0.1 [f]	53.7 ± 0.2 [g]
Polyunsaturated FA	18.9 ± 0.2 [a]	16.2 ± 0.1 [b]	17.0 ± 0.3 [c]	26.0 ± 0.3 [d]	25.4 ± 0.1 [d]	18.9 ± 0.3 [a]	12.7 ± 0.1 [e]

Table 4

Free fatty acids, mono- and diacylglycerols composition in semicontinuous cultivation (values are expressed as mg per grams of biomass dry weight) and saturation level (expressed as percentage with respect to total) of free fatty acids. In the same row, different letters indicate significant ($p<0.05$) differences, while identical letters indicate grouping of values with no significant ($p>0.05$) differences.

		N Replete		N Limited		N Deplete	
	Time 0	**Time 4**	**Time 7**	**Time 4**	**Time 7**	**Time 4**	**Time 7**
MAG (mg g^{-1})	$1.23 \pm 0.17^{a,b}$	1.46 ± 0.15^{a}	$2.52 \pm 0.23^{a,b}$	$3.67 \pm 0.3^{a,b}$	4.85 ± 0.4^{b}	$4.11 \pm 0.17^{a,b}$	22.32 ± 2.67^{c}
DAG (mg g^{-1})	0.97 ± 0.05^{a}	1.9 ± 0.27^{a}	2.13 ± 0.28^{a}	11.05 ± 1.13^{b}	14.3 ± 1.78^{b}	22.23 ± 1.89^{c}	46.62 ± 5.99^{d}
FFA (mg g^{-1})	2.85 ± 0.02^{a}	9.79 ± 0.08^{b}	6.04 ± 0.03^{c}	4.4 ± 0.04^{d}	7.05 ± 0.03^{e}	15.24 ± 0.11^{f}	57.28 ± 0.11^{g}
Saturated FFA	46.7 ± 0.5^{a}	38.5 ± 0.4^{b}	32.4 ± 0.2^{c}	54.9 ± 0.3^{d}	58.1 ± 0.1^{e}	54.5 ± 0.1^{d}	71.5 ± 0.2^{f}
Monounsaturated FFA	24.1 ± 0.3^{a}	37.9 ± 0.3^{b}	40.2 ± 0.2^{c}	30.4 ± 0.2^{d}	26.9 ± 0.1^{e}	37.4 ± 0.2^{b}	22.6 ± 0.1^{f}
Polyunsaturated FFA	29.2 ± 0.2^{a}	23.5 ± 0.7^{b}	27.5 ± 0.1^{c}	14.7 ± 0.1^{d}	15.0 ± 0.1^{d}	8.2 ± 0.1^{e}	5.9 ± 0.1^{f}

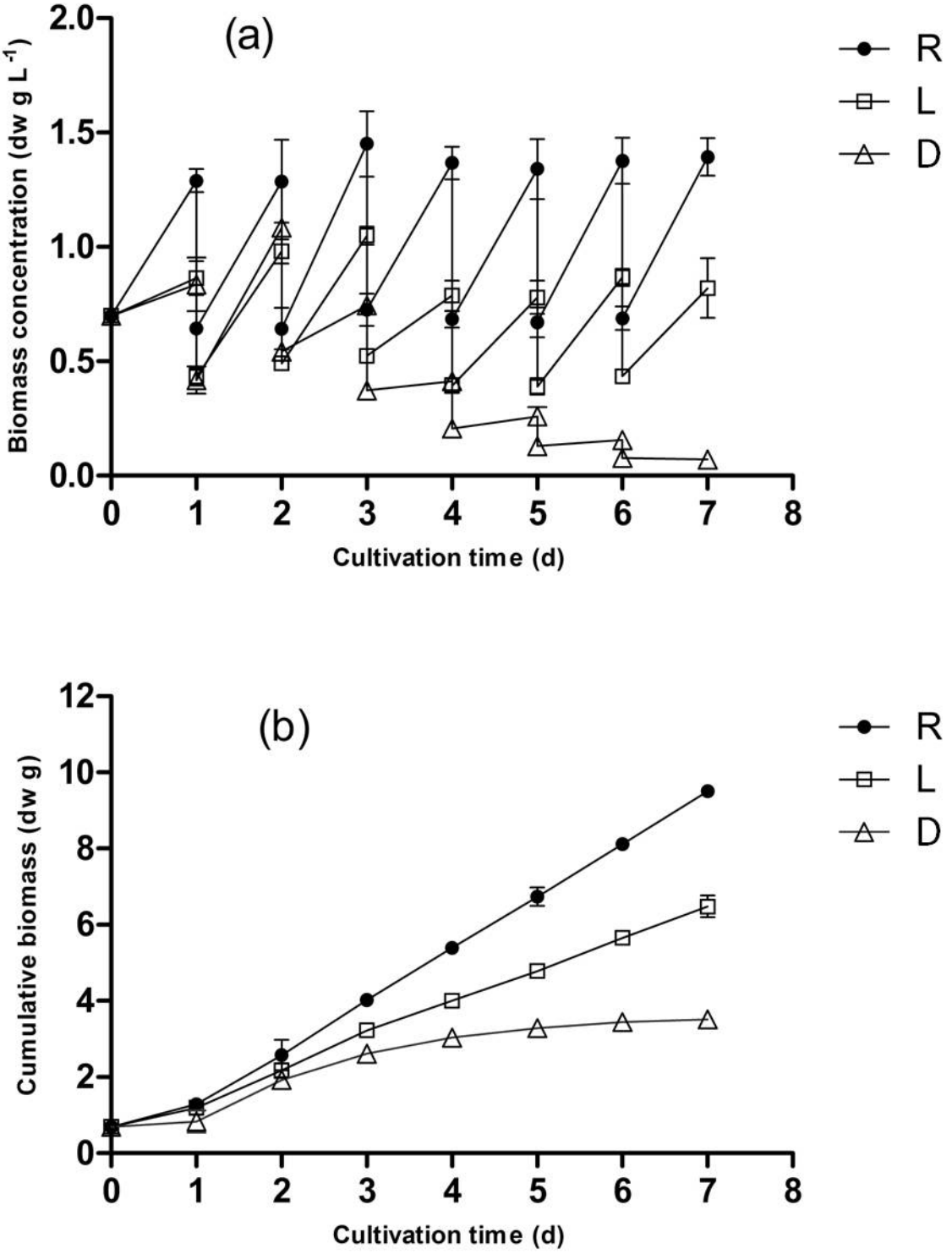

Fig. 1. a) Biomass concentration and b) cumulative biomass of replete (R), limited (L) and deplete (D) cultures

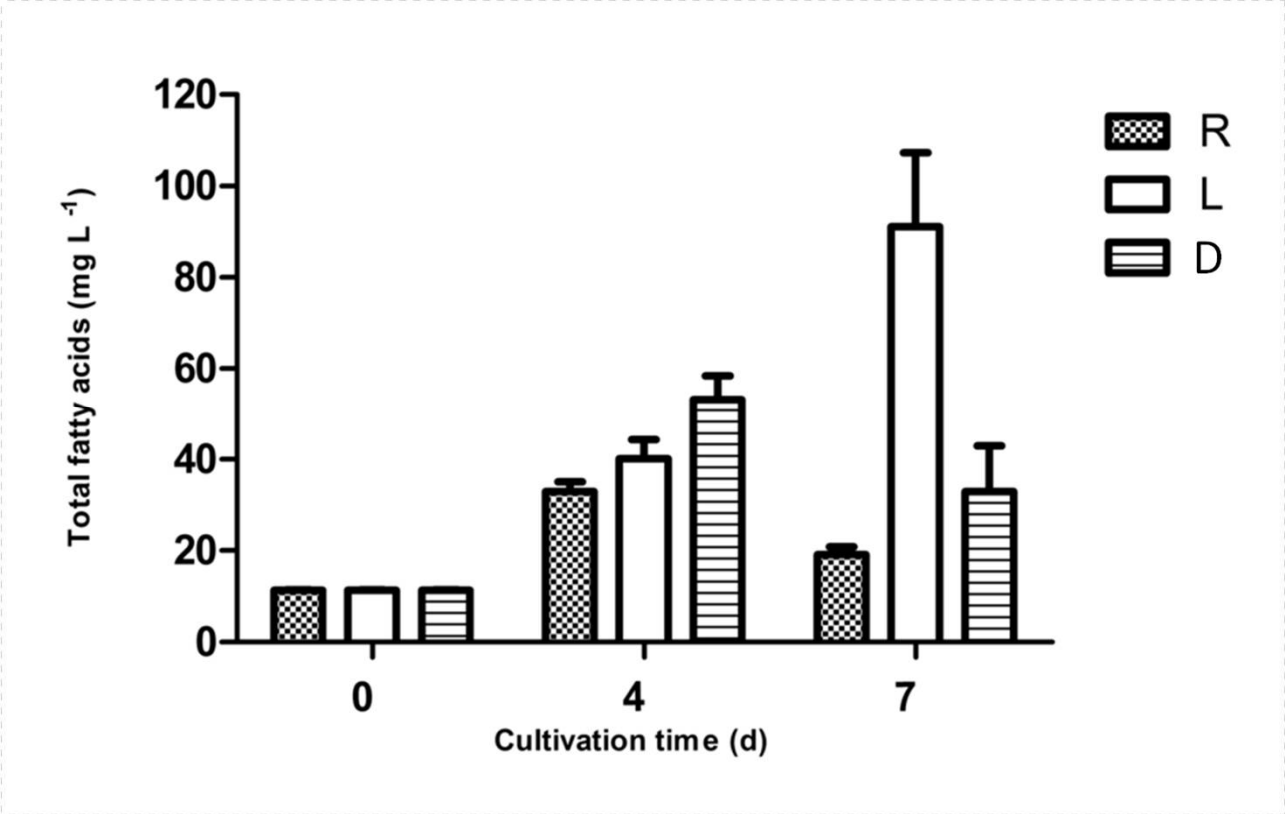

Fig.2. Total fatty acids (TFA) (mg L^{-1}) in replete (R), limitation (L) and deplete (D)